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Experimental Transmission of West Nile Virus by *Culex nigripalpus* from Honduras

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ABSTRACT

As a result of concerns regarding the geographic spread of West Nile virus (WNV) to Central America, we evaluated the potential for Honduran *Culex nigripalpus* Theobald to transmit this virus. We tested individual mosquitoes captured in Olancho Province, Honduras, in September 2003. Mosquitoes were allowed to feed on 2- to 4-day-old chickens previously inoculated with a New York strain (Crow 397-99) of WNV. Infection rates in *Cx. nigripalpus* ranged from 81%–96% after feeding on chickens with viremias between $10^{6.3}$ and $10^{7.4}$ plaque-forming units per milliliter. Development of a disseminated infection was directly correlated with holding time after the infectious blood meal as 68% (19/28) of the mosquitoes tested 20 days after the infectious blood meal had a disseminated infection as compared to 38% (15/40) of the mosquitoes tested 14 days after feeding on the same viremic chickens (viremia = $10^{6.9–7.4}$). Nearly all (4/5) *Cx. nigripalpus* with a disseminated infection that fed on susceptible chickens transmitted virus by bite. In addition, 8 (57%) of 14 *Cx. nigripalpus* with a disseminated infection transmitted virus when tested by a capillary tube feeding assay. Based on its efficiency of viral transmission in this study and its role in the transmission of the closely related St. Louis encephalitis virus in the southeastern United States, *Cx. nigripalpus* should be considered a potentially important vector of WNV in Honduras and the rest of Central America. Key Words: *Culex nigripalpus*—West Nile virus—Vector competence—Honduras.
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INTRODUCTION

SINCE ITS FIRST RECOGNITION in the New York metropolitan area in 1999 (Centers for Disease Control and Prevention [CDC] 1999, Lanciotti et al. 2000), West Nile virus (WNV) has expanded its range in the United States. By September 2002, its range included the entire eastern seaboard, and it had spread westward to California, and southward into the Caribbean (CDC 2002a, 2002b). Throughout this range, WNV has been isolated from birds, principally crows, and has been responsible for encephalitis and death in humans and horses (CDC 2001).

WNV is a member of the Japanese encephalitis virus serogroup in the genus *Flavivirus*, family *Flaviviridae*, and is closely related to St. Louis encephalitis virus (SLEV), also a member of this serogroup. The viruses share a

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14. ABSTRACT

Due to concerns regarding the geographic spread of West Nile virus (WNV) to Central America, we evaluated the potential for Honduran Culex nigripalpus Theobald to transmit this virus. We tested individual mosquitoes captured in Olancho Province, Honduras, in September 2003. Mosquitoes were fed upon 2- to 4-day-old chickens previously inoculated with a New York strain (Crow 397-99) of WNV. Infection rates in Cx. nigripalpus ranged from 81-96% after feeding on chickens with viremias between 106.3 and 107.4 plaque-forming units (PFU)/ml of blood. Development of a disseminated infection was directly correlated with holding time after the infectious blood meal, as 74% of the mosquitoes tested 20 d after the infectious blood meal had a disseminated infection as compared to 42% of the mosquitoes tested 14 d after feeding on the same viremic chicken. Nearly all (86%) of Cx. nigripalpus with a disseminated infection that fed on susceptible chickens transmitted virus by bite. In addition, 8 (57%) of 14 Cx. nigripalpus with a disseminated infection transmitted virus when tested by a capillary tube feeding assay. Based on its efficiency of viral transmission in this study and its role in the transmission of the closely related St. Louis encephalitis virus in the southeastern USA, Cx. nigripalpus should be considered a potentially important vector of WNV in Honduras and the rest of Central America.

15. SUBJECT TERMS
West Nile virus, mosquito vector, Culex nigripalpus, transmission, field study, Honduras

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similar epidemiology. The natural transmission cycle for both of these viruses is between ornithophilic mosquitoes, principally members of the genus *Culex*, and various avian hosts. Because of the potential for WNV to spread into more southerly regions, and the previous finding that *Culex nigripalpus* Theobald was a competent vector of WNV in the United States (Sardelis et al. 2001), there is concern that this species might be an important vector of WNV in Central America, where *Cx. nigripalpus* is abundant (Turell et al. 2003). Thus, we evaluated the potential for *Cx. nigripalpus* from Honduras to become infected with and transmit WNV under laboratory conditions.

MATERIALS AND METHODS

Culex nigripalpus, collected in dry ice-baited miniature light traps (John W. Hock Co., Gainesville, FL) during September 2003 in Olancho Province, Honduras, were transported to a biologic safety level 3+ laboratory at the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland. These mosquitoes were allowed to feed on an uninfected hamster, and their F_1 progeny were used in the following studies. Adult mosquitoes were kept in 3.8-liter cartons, with netting over the open end, placed in an incubator maintained at 26°C with a 16:8 hour photoperiod, and provided with apple slices and water-soaked gauze pads. Groups of 20–50 female mosquitoes were transferred to 0.9-liter cardboard cartons with netting over the open end. Carbohydrates were withheld for 1 day before mosquitoes were exposed to WNV by allowing them to feed on 2- to 4-day-old chickens that had been inoculated with 10^4 plaque-forming units (PFU) of WNV when they were 1 day old. Immediately after mosquito feeding, a 0.1-mL blood sample was obtained from the jugular vein of each chicken and diluted in 0.9 mL of diluent (10% heat-inactivated fetal bovine serum in Medium 199 with Earle's salts, $NaHCO_3$, and antibiotics) plus 10 units of heparin per milliliter. This sample was frozen at −70°C until tested by plaque assay to determine the viremia at the time of mosquito feeding. Engorged mosquitoes were transferred to 3.8-liter cages and maintained in an incubator at 26°C as described above.

To determine the percentage of *Cx. nigripalpus* that could transmit WNV by bite, we allowed the *Cx. nigripalpus* to feed again (12–14 or 19–21 days after the infectious blood meal) either individually or in pairs on unexposed 1- to 2-day-old chickens. The chickens were bled the following day to determine if they became infected. Because some of the mosquitoes were tested for transmission in pairs, it was not always possible to determine which mosquito in a pool actually transmitted virus by bite. Therefore, if more than one mosquito with a disseminated infection fed in a pool, data from that pool were not used to calculate the transmission rate, regardless of chicken viremia. Some of the mosquitoes were also tested for their ability to transmit virus to diluent in a capillary tube. Briefly, mosquitoes were chilled in a glass container in wet ice. Their legs were removed and triturated for virus testing, their wings removed, and their bodies placed on their sides on sticky tape. A glass capillary tube containing about 10 μL of diluent (fortified to 50% heat-inactivated fetal bovine serum) was placed so that the mosquito's proboscis was inserted into the diluent. Thirty minutes later, the diluent was expressed into 500 μL of diluent and the mosquito's body was triturated for virus testing. The diluent, presumably containing the expressed saliva, was tested by plaque assay on 6-well plates in triplicate for the presence of virus.

To determine infection and dissemination rates, we killed the mosquitoes by freezing them at −20°C for 5 minutes (13–15 or 19–21 days after the infectious blood meal) and triturated their legs and bodies separately in 1 mL of diluent. These suspensions were stored at −70°C until tested for virus by plaque assay. Presence of virus in a mosquito's body indicated infection, while virus in the legs indicated the mosquito had a disseminated infection (Turell et al. 1984).

Throughout the study, we used the Crow 397-99 strain of WNV. This strain was isolated from a dead crow found in the Bronx, New York City, during an epizootic in 1999 (Turell et al. 2000) and had been passaged once in Vero cell culture. Viral stocks, triturated mosquito

suspensions, mosquito salivary suspensions, and chicken blood samples were tested for infectious virus by plaque assay on African green monkey kidney (Vero) cells as described by Gargan et al. (1983), except that the second overlay, containing neutral red stain, was added 2 days after the first overlay.

We defined the infection rate as the number of infected mosquitoes/total tested × 100 and the dissemination rate as the number of mosquitoes with virus in their legs/total tested × 100. We used Intercooled Stata 7.0 (Stata Corporation, College Station, TX) to calculate exact 95% confidence limits for infection and dissemination rates and to calculate levels of significance for differences in these values. To estimate transmission rates, we multiplied the percentage of mosquitoes that developed a disseminated infection after ingesting WNV by the percentage of mosquitoes with a disseminated infection that transmitted virus by bite.

RESULTS AND DISCUSSION

Viremias at the time of mosquito feeding ranged from $10^{6.3}$ to $10^{7.4}$ PFU/mL of blood. Virtually all (65/68) of the *Cx. nigripalpus* that ingested blood from chickens with high viremias ($10^{6.9–7.4}$ PFU/mL) became infected (Table 1). In general, *Cx. nigripalpus* that fed on chickens with viremias of $10^{6.9–7.4}$ PFU/mL had higher infection and dissemination rates than did those that fed on chickens with viremias of $10^{6.3–6.7}$ PFU/mL (Table 1). Although infection rates were relatively constant at the two times tested, dissemination rates were significantly higher at 20 days as compared to those tested at 14 days for those mosquitoes that fed on chickens with viremias of $10^{6.9–7.4}$ PFU/mL ($\chi^2 = 6.07$, $df = 1$, $p = 0.014$). Infection rates were significantly higher in those mosquitoes (days 14 and 20 combined) that ingested $10^{6.9–7.4}$ PFU/mL, than those that ingested $10^{6.3–6.7}$ PFU/mL (Fisher's exact $p = 0.044$). The length of the extrinsic incubation period did not influence infection rates (Fisher's exact $p = 0.43$), but it did have a significant effect on dissemination rates ($\chi^2 = 7.22$, $df = 1$, $p = 0.007$) between cohorts of mosquitoes held for 14 or 20 days, respectively (exposure doses combined). Nearly all (4/5) of the WNV-exposed *Cx. nigripalpus* that fed individually on uninfected chickens transmitted virus by bite. In addition, there were three pairs of *Cx. nigripalpus* that each fed on a single chicken. In each case, the chicken became viremic. However, these data could not be used in calculating the transmission rate. In comparison, 8 of 14 (57%) of *Cx. nigripalpus* with a disseminated infection

TABLE 1. SUSCEPTIBILITY OF *CULEX NIGRIPALPUS* TO WEST NILE VIRUS AFTER FEEDING ON VIREMIC CHICKENS

Criteria	*Days after the infectious blood meal*	
	14 ± 1	*20 ± 1*
Fed on chickens with a mean viremia of $10^{6.3–6.7}$		
Number tested	26	1
Infection rate[a]	81 (62,94)	100 (2,100)
Dissemination rate[b]	33 (17,54)	0 (0,98)
Estimated transmission rate	26	—
Fed on chickens with a mean viremia of $10^{6.9–7.4}$		
Number tested	40	28
Infection rate[a]	95 (83,99)	96 (82,100)
Dissemination rate[b]	38 (23,54)	68 (48,84)
Estimated transmission rate[c]	30	54

[a]Percentage of mosquitoes containing virus in their bodies (95% confidence interval).
[b]Percentage of mosquitoes containing virus in their legs (95% confidence interval).
[c]The estimated transmission rate = the percentage of mosquitoes that developed a disseminated infection after ingesting WNV multiplied by the transmission rate for those individuals with a disseminated infection (see Table 2).
WNV, West Nile Virus.

transmitted virus when tested by a capillary tube feeding assay (Table 2). Although these differences were not significant (Fisher's exact test, $p = 0.33$), they are very similar to those obtained with *Cx. tritaeniorhynchus* and WNV; 93% of 69 *Cx. tritaeniorhynchus* with a disseminated infection transmitted WNV when fed on a suckling mouse, but only 61% of 126 transmitted WNV to a hanging drop (Akhter et al. 1982). Another study similarly concluded that transmission rates to animals were more sensitive than capillary tubes, where 100% of 20 *Cx. univittatus* transmitted WNV to hamsters and only 78% of 28 mosquitoes transmitted WNV to capillary tubes (Cornel and Jupp 1989). Therefore, transmission rates obtained with the capillary method may be an under estimate of the true transmission rate. This apparent under estimate might be overcome by use of a more sensitive virus detection system, such as animal inoculation versus cell culture (Smith et al. 2005). However, this would not reduce experimental animal numbers, which is the most compelling reason for capillary tube feeding assay use. Because there does not appear to be a significant salivary gland barrier for WNV in *Cx. nigripalpus*, we expect about 54% of *Cx. nigripalpus* would be able to transmit WNV by day 20 after oral exposure to $10^{6.9-7.4}$ PFU/mL of WNV. However, after 14 days of incubation, we would expect only 30% of *Cx. nigripalpus* to transmit WNV.

This study showed that a Honduran strain of *Cx. nigripalpus* has the potential to serve as a vector for WNV based on its susceptibility to infection and ability to transmit WNV. This finding is consistent with previous laboratory transmission studies that showed that WNV is transmitted by a variety of North American mosquito species, including a number of *Culex* and *Aedes* species (Turell et al. 2000, 2001, Sardelis and Turell 2001, Sardelis et al. 2001). Unlike most of the other North American *Culex* (*Culex*) species tested (Turell et al. 2000, 2001, Sardelis et al. 2001), there was little evidence of a midgut escape barrier in *Cx. nigripalpus*. However, this may have been due to testing many of the specimens at 20 days in this study compared to 14 days in the previous studies. Also, because nearly all individuals with a disseminated infection tested transmitted WNV by bite, there appeared to be no salivary gland barrier for WNV in this species. Although *Cx. nigripalpus* appears to be a moderately efficient laboratory vector of WNV, various aspects of the mosquito's bionomics must be considered to effectively evaluate the importance of this species in the transmission of WNV in nature. *Culex nigripalpus* is considered an ornithophilic feeder, taking blood meals mostly from birds, but feeding on mammals becomes more common in late summer (Edman and Taylor 1968). In addition, it is considered to be both the maintenance and epidemic vector of SLEV in the southern United States (Chamberlain and Sudia 1964).

The viremias used in our study, $10^{6.3-7.4}$ PFU/mL of blood, are consistent with low to moderate viremias for hooded crows and

TABLE 2. TRANSMISSION OF WEST NILE VIRUS BY *CX. NIGRIPALPUS* AFTER FEEDING ON VIREMIC CHICKENS

Method	Viremia	Transmission rate[a]			Transmission rate (D)[b]
		14 ± 1 d	20 ± 1 d	Totals	
Chicken	$10^{6.3-6.7}$	100 (1)	n.t.	100 (1)	100 (1)
	$10^{6.9-7.4}$	43 (7)	n.t.	43 (7)	75 (4)
	Subtotal	50 (8)	n.t.	50 (8)	80 (5)[c]
Capillary	$10^{6.3-6.7}$	n.t.	0 (1)	0 (1)	n.t.
	$10^{6.9-7.4}$	0 (8)	47 (17)	33 (24)	57 (14)
	Subtotal	0 (8)	44 (18)	32 (25)	57 (14)

[a]Percentage of mosquitoes that transmitted by bite (number fed).
[b]Percentage of mosquitoes with a disseminated infection that transmitted virus by bite (number fed).
[c]In addition, there were 3 cases in which 2 mosquitoes with a disseminated infection fed on the same chicken. In all 3 cases the chicken became infected. These data are not included in the table.

house sparrows in Egypt (Work et al. 1955) and experimentally infected North American house sparrows and crows (Komar et al. 2003). Thus, our results should reflect what would happen when *Cx. nigripalpus* fed on birds with a similar concentration of virus in nature. Based on the recent findings of WNV strains with altered phenotypes, further investigation of the intraspecific differences in viremia, and ultimately *Cx. nigripalpus* vector competence due to variation in WNV strain virulence is warranted (Davis et al. 2004).

The results of this study, combined with evidence of the distribution and bionomics of *Cx. nigripalpus*, suggest that this species could function both as a maintenance as well as a bridge vector for WNV. Due to intraspecific variation in vector competence, our use of a regionally appropriate strain of *Cx. nigripalpus* improved our ability to estimate the role this species may play in the epidemiology of WNV in Honduras. However, further evaluation of more regionally appropriate strains of WNV in *Cx. nigripalpus* from Honduras is indicated as such virus isolates are identified.

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